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GENETIC REGULATION OF LIPID BIOGENESIS IN HUMAN BREAST CANCER

PI: James M. Ntambi

(1) INTRODUCTION

Cancer is a debilitating disease and a leading cause of death worldwide characterized by increased and uncontrolled cell growth. To accommodate their increased rates of proliferation, cancer cell membranes are typically less stable and more fluid than those of benign cells (Ntambi, 1999). The increased fluidity of the membranes may be a requirement for increased cellular proliferation, growth, and metabolism. Assessing mechanisms to normalize membrane fluidity in cancer cells may offer great therapeutic potential for cancer treatment .

Stearoyl-CoA desaturase (SCD) is an oxidative enzyme crucial for the synthesis of unsaturated fatty acids. SCD inserts a *cis* double bond between the 9th and 10th carbons in the saturated fatty acids palmitoyl-CoA (16:0) and stearoyl-CoA (18:0) to produce palmitoleic (16:1) and oleic (18:1) acids, respectively. The monounsaturated products can then act as substrates for the synthesis of numerous cellular lipids including triglycerides, phospholipids, wax esters, and cholesterol esters. Because SCD activity increases the cellular ratio of unsaturated to saturated fatty acids, cells expressing higher levels of SCD are likely to have a higher unsaturated fatty acid component to their membranes, increasing membrane fluidity and permeability.

Both *in vivo* and *in vitro* regulation of SCD by intermediates, products, and mediators of lipid metabolism have been relatively well studied. For example, the sterol regulatory element binding proteins (SREBPs) activate expression of SCD in response to changes in cholesterol levels (Tabor, 1999). Polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 categories and other lipids such as cholesterol have been shown to repress SCD promoter activity, mRNA, and protein levels in a variety of cell types (Jeffcoat, 1978; Waters, 1997; Tabor, 1998; Ntambi, 1999; Tabor, 1999;

Bené, 2001). However, PUFA and cholesterol regulation of SCD in human cancer cells has not been well characterized. Because cancer cells require elevated levels of lipogenesis and increased membrane fluidity, understanding SCD's expression and regulation in cancer may provide vital therapeutic answers to questions regarding cancer treatment.

This study employs MCF-7 and MDA-MB-231 (MDA) human breast cancer cell lines to examine differences in SCD regulation in cancer cells. MCF-7 cells express two wild-type alleles for the p53 tumor suppressor while MDA cells contain a loss-of-function mutation in the DNA binding domain of p53 (Xu, 2001). Human p53 is a 393 amino acid, four-domain protein that induces apoptosis, represses anti-apoptotic genes, and / or induces growth arrest in the G_1 phase of the cell cycle in response to cellular stress such as DNA damage. Mutations in p53, which occur in over 50% of human cancers, increase the likelihood of unregulated cellular proliferation and eventual tumor formation by inhibiting cellular response to distress signals (Chen, 1996; Weinberg, 1996; Levine, 1997; Hoffmann, 2002).

The proteins involved in the direct expression of SCD may help explain its regulation. The 57 base-pair polyunsaturated fatty acid response element (PUFA-RE) is a necessary promoter region for PUFA regulation of SCD (Figure 1; Waters, 1997; Bené, 2001). Four known transcription factors interact with the PUFA-RE, including SP-1, the SREBPs, NF-Y, and NF-1. In addition to mediating the cholesterol regulation of SCD, the SREBPs regulate the PUFA response of SCD and other lipogenic genes. Nuclear factor Y (NF-Y) and SP-1 are important in the PUFA mediated repression of fatty acid synthase (FAS), another lipogenic gene, via its PUFA-RE (Clarke, 2001). NF-Y is also involved in p53-induced cell cycle arrest and cellular senescence (Yun, 1999; Jung, 2001; Manni, 2001). Therefore, based on similarities between the downstream effects of p53, the regulation of SCD, and the importance of SCD and its associated transcription factors in lipid metabolism and cancer cell proliferation, it becomes possible that p53 may regulate SCD as a means of controlling cell growth.

Originally we hypothesized that polyunsaturated fatty acids (PUFA) inhibit cell proliferation associated with fatty acid synthesis by regulating the interaction of the mutant p53

protein with the specific motif on the promoters of the genes involved in fatty acid biosynthesis. We have found that the wild-type p53 protein significantly represses the transcription of the stearoyl-CoA desaturase gene (SCD) through the 57bp regulatory element we previously termed polyunsaturated fatty acid responsive element (PUFA-RE) (Waters, 1997). The PUFA-RE contains the SRE and CCAAT sequences known to mediate the transcriptional activation of lipogenic genes by the sterol regulatory element binding protein (SREBP) and NF-Y, respectively. Previous studies (Tabor, 1999) had proposed that by binding to the SRE and CCAAT, SREBP and NF-Y mediate PUFA-repression of lipogenic genes. We speculated that p53 might repress the transcription of the SCD1 gene through the same sequences. When we mutated the SRE and a sequence between the SRE and NFY, we did not abolish the p53-mediated repression of the SCD promoter. However when the NFY and NF-1 sequences (Fig. 1) were mutated, p53-mediated repression was abolished. These results suggest that a novel sequence the gccaatggca that comprises the NFY and NF-1 binding sites is responsible for p53-mediated repression of the SCD1 gene. In the presence of a dominant negative mutant of the NF-YA subunit the p53 does not decrease the promoter activity suggesting that the NF-Y transcription factor is required for the p53-mediated regulation.

(2) BODY

Materials and Methods

Cell Culture. MDA and MCF-7 human breast cancer cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin / streptomycin,

and 0.1% fungizone and incubated at 37 °C in 5% CO_2. Media was changed every 2 – 3 days, and cells were split upon reaching confluency.

Doxorubicin Treatments. Adriamycin (a.k.a. doxorubicin, Sigma-Aldrich) is a chemical inducer of p53, which inhibits cell growth and induces apoptosis in MCF-7 cells (Burrow, 2001; Chen, 2002). At nearly 100% confluency, MCF-7 cells were treated with varying concentrations of doxorubicin (DOX) dissolved in water. Approximately 24 hours after treatment, total cellular protein or cellular microsomes were isolated as described below.

Vector Construction. The Biotechnology Center of the University of Wisconsin – Madison synthesized the 57 base-pair (bp) wild-type sequence of the human PUFA-RE, 330-386 bp upstream of the transcription start site of the SCD1 gene. Several mutant PUFA-RE oligos were also created by mutating a region of bases between A↔C and T↔G (Figure 1). All oligonucleotides contained a BamHI sticky end on the 5' end and a HindIII sticky end on the 3' end. The inserts were cloned into BamHI / HindIII digested pTK-Luc vector (ATCC), which has a multicloning site upstream of a TK promoter and a *Firefly* luciferase reporter gene. All restriction and modification enzymes were from Promega in Madison, WI.

DNA Transfections. MDA and MCF-7 cells were cultured as described above in 60mm plates to approximately 50% confluency. 5 μg of the promoter luciferase constructs were transfected with 250 ng of phRL-null vector (Promega) as a control using Transit LT-1 Reagent (Mirus). In co-transfection experiments, 100ng of empty pCMV-NEO-BAM vector, vector containing a loss-of-function mutated p53 gene (248: O→S) (p53 mut), or vector containing wild-type p53 DNA (p53 w/t) was added to determine p53's effect on luciferase construct activity. After 48 hours of incubation at 37°C supplemented with 5% CO_2, cells were lysed using passive lysis buffer and *Firefly* and *Renilla* luciferase counts were read using Promega's Dual Luciferase Assay, and protein concentrations were determined using a Bio-Rad Bradford Assay (Bradford, 1976). Expression levels of the given promoters were normalized against a negative control.

Western Blotting & Analysis. Total cellular protein samples were extracted and isolated using the RIPA lysis method. Protein concentrations were determined using a Bio-Rad Bradford

Assay (Bradford, 1976), and 40 μg of protein was electrophoresed on a denaturing 10% acrylamide gel with an appropriate protein marker. The gel was transferred to a nitrocellulose membrane and blocked overnight at 4° C in BSA blocking buffer. The membranes were incubated with the appropriate primary antibody for 1 hour followed by the appropriate secondary antibody for 30 minutes. Blots were exposed using the ECL protein detection method.

Microsome Isolation & SCD Activity Assay. Twenty-four hours after treatment with DOX, MCF-7 cells were washed twice with cold (4°C) PBS and then once with cold (4°C) 10mM Tris, pH 7.4, containing 1 mM dithiothreitol and 0.25 M sucrose (TDS). The cells were resuspended in 1 mL of cold TDS and collected with a cell scrapper. The cells were homogenized using a variable-speed polytron tissue disruptor (Biospec Products) and then centrifuged at 15,000 g for 20 min at 4°C. The cell pellet was discarded, and the supernatant was spun in an ultracentrifuge at 100,000 g for 1 h at 4°C. The supernatant was discarded, and the microsome pellet was resuspended in 100 μL of 0.1 M sodium phosphate buffer, pH 7.4. Protein was quantitated with a Bradford Assay (Bradford, 1976), and SCD activity was measured as previously described (Gomez, 2002).

(3) RESULTS

To investigate further the hypothesis of p53-mediated repression of SCD, MCF-7 cells were treated with doxorubicin (DOX), a known chemical inducer of DNA damage and the p53 pathway. DOX had been previously shown to safely induce p53 in MCF-7 cells (Hoffmann, 2002). Prior to treatment with DOX, MCF-7 cells do not show significant expression of p53, but p53 levels clearly increase upon DOX exposure (Figure 2). Levels of SCD protein decrease with p53 induction in MCF-7 cells while tubulin levels remain steady throughout DOX treatments, indicating that DOX does not affect whole-cell protein levels. Furthermore, treatment with DOX significantly decreases SCD activity as measured by tritiated-water release from stearoyl-CoA in the presence of microsomes from DOX-treated MCF-7 cells (Figure 2B).

This data suggest a role for p53 as a transcription factor on SCD and provide substantial evidence for a p53-dependent repression of SCD in human breast cancer cells.

We previously demonstrated (reports 1 and 2) that the PUFA-RE of the SCD1 gene was important for p53 repression of the SCD1 gene. This was accomplished by cloning the 60 bp. PUFA-RE region into a pTK-luc vector and transfected into MCF-7 cells with empty pCMV-NEO-BAM vector, p53 mut, or p53 w/t DNA. The presence of p53 w/t DNA repressed SCD promoter activity to a degree similar to p53's effect on the entire –570-luciferase construct.

Given the relationship between p53 repression of SCD and the PUFA-RE, mutations of the PUFA-RE were created to isolate the site of p53 activity (Figure 1). It was thought that drastic alterations to its site of action would cause a loss of p53 function as indicated through repression of hSCD promoter activity. Functional p53 w/t DNA represses wild type PUFA-RE promoter activity despite mutations in the binding sites for the SREBP transcription factor and SP-1 (Figure 3A). A mutation in the SRE and part of NF-Y binding sites slightly reduces p53-mediated repression while a mutation corresponding to the region comprising the NF-Y and the NF-1 binding sites results in complete loss of repression (Fig. 3A). These results suggest that a novel sequence gccaatggca (Fig. 1) is responsible for p53-mediated repression of the SCD1 gene. To determine whether the NF-Y is one of the transcription factors is involved in the p53-mediated repression of the SCD promoter a dominant negative mutant of the NF-YA subunit was cotransfected with the PUFA-RE and p53w/t and the luciferase activity was assayed 24h later. Figure 3B shows that in the presence of the dominant mutant of NF-YA subunit, p53 did not decrease the promoter activity. These results suggest the NF-Y transcription factor is one of the factors required for the p53-mediated repression of the SCD gene.

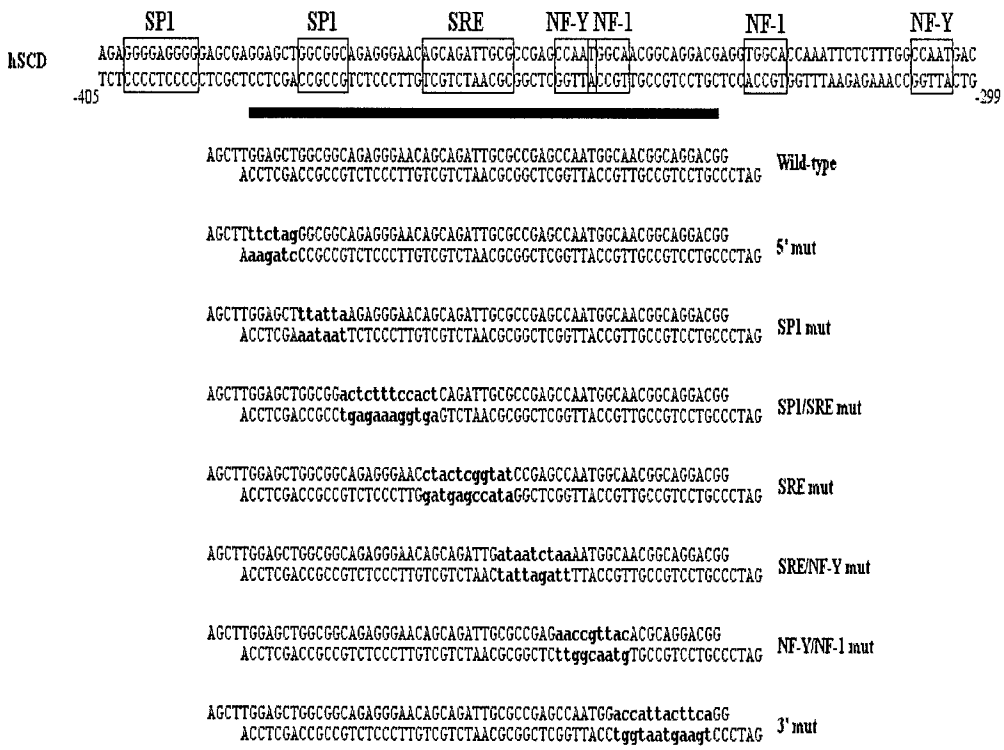


Figure 1 - The highlighted sequence indicates the PUFA-RE portion of the hSCD promoter. The mutated sequences used to determine the site of p53 activity on SCD are shown in lower case font. The boxes indicated known binding sites for transcription factors. The binding sites are labeled according to their corresponding protein with the exception of the sterol response element (SRE), which binds SREBPs. This study did not use all of these mutated sequences.

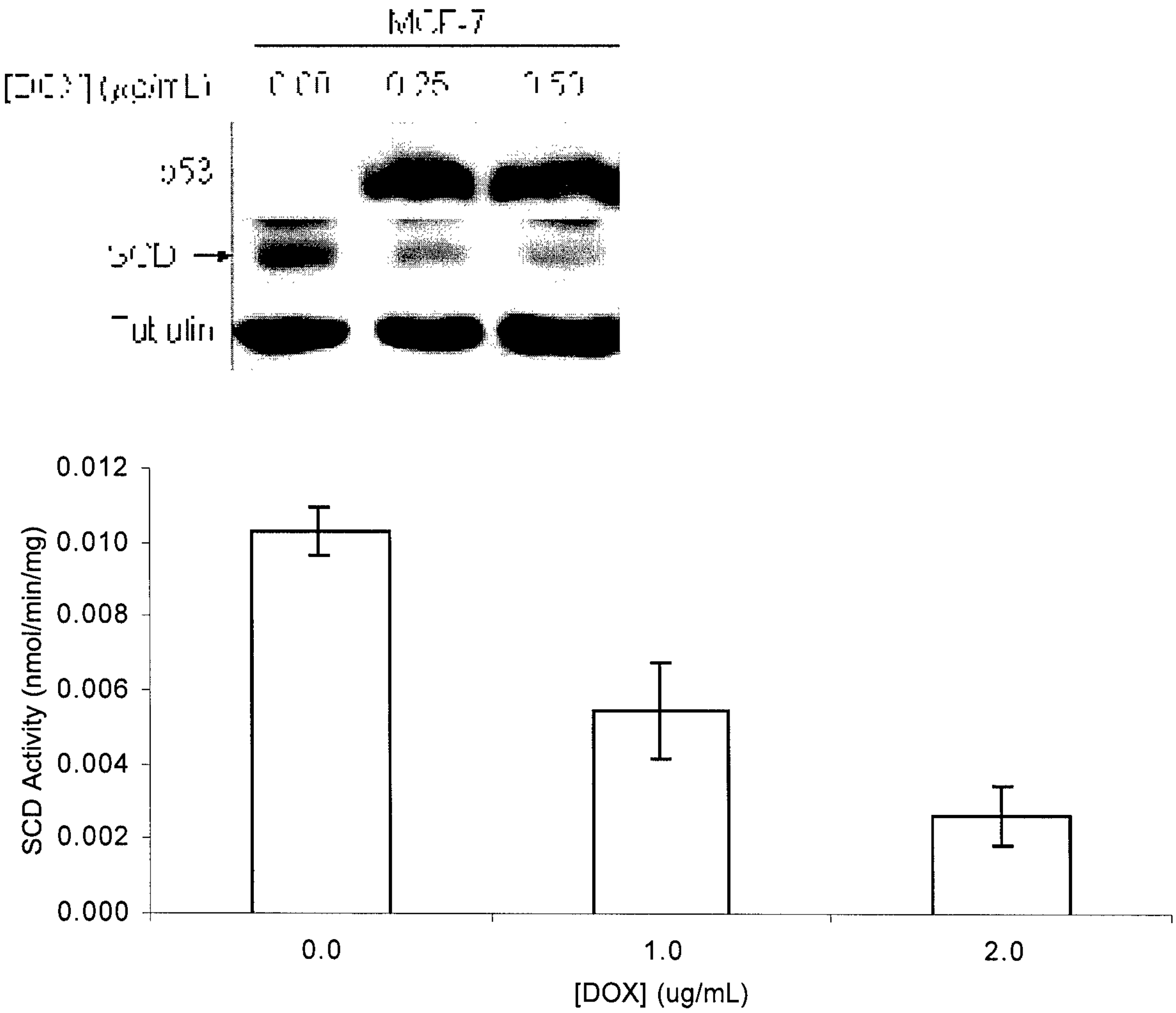
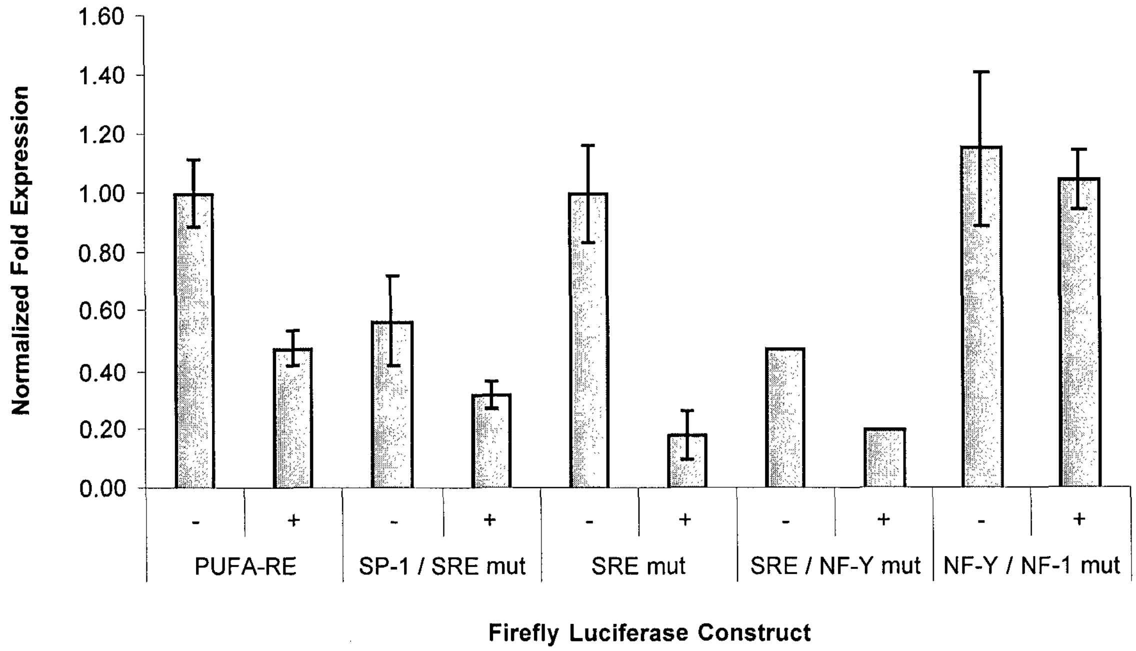


Figure 2 – Doxorubicin (DOX) mediated induction of p53 reduces SCD protein expression (A) and SCD activity (B) in MCF-7 cells. Tubulin was used as a loading control.

A



B

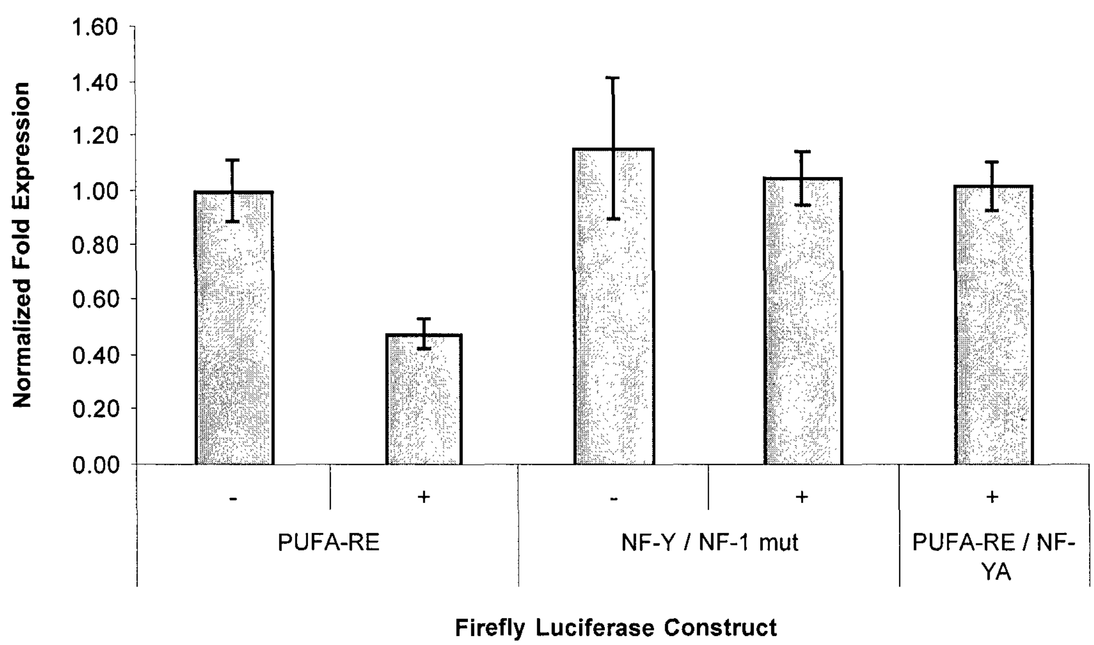


Figure 3 – A. MCF-7 cells were co-transfected with 5 μg Firefly luciferase construct and 100 ng empty pCMV-NEO-BAM vector (-) or vector containing p53 wild type DNA (+). p53 reduces promoter activity on the wild type PUFA-RE, SP-1/SRE-mut, SRE/mut and SRE/NF-Y-mut,

construct. Repression is lost with a mutation in the region comprising of NFY and NF-1 binding sites. Expression levels for all constructs were normalized to the p53$^-$ control. Protein levels as measured with a Bradford assay corrected firefly sums.

B. MCF-7 cells were co-transfected with 5 μg PUFA-RE and 100 ng of p53 wild type and NF-YA (the dominant negative subunit of NF-YA subunit) DNA. p53 does not reduce promoter activity in the presence of NF-YA. Expression levels for all constructs were normalized to the p53$^-$ control.

(5) KEY RESEARCH ACCOMPLISHMENTS:

1. We have obtained further evidence of p53-mediated repression of the SCD gene expression by treating MCF-7 cells with doxorubicin (DOX), a known chemical inducer of DNA damage and the p53 pathway. Prior to treatment with DOX, MCF-7 cells do not show significant expression of p53, but p53 levels clearly increase upon DOX exposure (Figure 2). Treatment with DOX significantly decreases SCD activity as measured by tritiated-water release from stearoyl-CoA in the presence of microsomes from DOX-treated MCF-7 cells (Figure 2B). This data clearly shows that the decrease in SCD1 gene transcription observed when the SCD-1 promoter was transfected with the wt p53 expression construct correlates with decrease in SCD-1 activity and providing substantial evidence for a p53-dependent repression of SCD in human breast cancer cells.

2. The SRE and partial NF-Y sequences within the PUFA-RE were previously mutated individually (report 2) and yet p53 still repressed expression of the SCD1 promoter activity, suggesting another site within the PUFA-RE was the target for p53-mediated repression.

3. By performing finer mapping and mutating the region comprising the NF-Y and NF-1 of the PUFA-RE we discovered the sequence is responsible for the p53-mediated repression of the SCD1 gene.

4. By using the dominant negative form of NF-Y to deplete endogenous NF-Y protein, we demonstrated that the p53-mediated repression of the SCD1 promoter activity requires NF-Y transcription factor. A similar experiment using the dominant negative form of NF-1 will have to be performed to determine whether the NF-1 transcription factor is also required in the p53-mediated repression of the SCD gene. A possibility exists that a complex of proteins is required to mediate repression.

(6) REPORTABLE OUTCOMES

Henry Bené, David Lasky, and **Ntambi, J. M.** (2001) The Cloning and Characterization of the Human Stearoyl-CoA Desaturase Gene Promoter: Transcriptional Activation by Sterol Regulatory Element Binding Protein and Repression by Polyunsaturated Fatty Acids and Cholesterol. BBRC 284, 1194-8

Youngjin Choi, Yeonhwa Parka, Jayne M. Storkson, Michael Pariza and **James M. Ntambi** (2002) Inhibition of stearoyl-CoA Desaturase activity by the cis-9, trans-11 isomer and the trans –10, cis-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells BBRC 294, 785-790.

Michael W. Cullen, Nicholas J. Ansay, Yudi A. Soesanto David A. Lasky, and **James M. Ntambi**. The Role of Polyunsaturated Fatty Acids, Cholesterol, and p53 in the Regulation and Expression of Stearoyl Co-A Desaturase in Human Breast Cancer Cell Lines Manuscript in Preparation

(7) CONCLUSIONS

This study has succeeded in isolating the effects of p53 on human SCD to the PUFA-RE and determining a specific site for p53 action. That p53 acts to regulate SCD in part through the PUFA-RE provides further support for the hypothesis that PUFA-dependent regulation of SCD may be related to p53. The importance of the NF-Y binding site to p53 action is also critical to understanding p53 regulation of SCD. As described earlier, NF-Y, or the CCAAT binding protein transcription factor, mediates numerous downstream effects in the p53 pathway. Perhaps these results suggest that SCD is simply another target for p53's effects on cellular growth and metabolism as mediated by NF-Y or NF-1. However, it is also possible that a novel protein binds to the region of the PUFA-RE made up of the NF-Y and NF-1 sites and further studies will be needed to address this possibility.

A significant finding of this study involves the decrease in SCD protein levels and activity in response to p53 induction by doxorubicin. p53's activity as a powerful regulator of cell-cycle progression and apoptosis has been very well characterized, but it had never been shown to directly affect genes involved in lipid metabolism, particularly SCD. Nevertheless, the physiological theory for this interaction is sound. Cancer cells are thought to require increased levels of lipid synthesis to support their increased growth and metabolic rates (Ntambi, 1999).

Therefore, induction of the p53 pathway should down-regulate lipogenic gene expression to decrease cellular growth rates.

In conclusion, this study successfully characterizes many previously unexamined aspects of stearoyl Co-A desaturase regulation in MDA and MCF-7 human breast cancer cell lines. It also raises several new questions regarding interactions between the p53 tumor suppressor, other lipogenic genes, and numerous intermediates and products of lipid metabolism. It is hoped that the questions addressed will provide impetus for future studies regarding interactions between tumor suppressors and lipogenic genes. These studies may develop new and creative ways to fight cancer, diabetes, and other diseases associated with cell growth and lipid metabolism.

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REPLY TO
ATTENTION OF

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FOR THE COMMANDER:

Phylis Rinehart

PHYLIS M. RINEHART
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